Increased Oxidant-Induced Apoptosis in Cultured Nondividing Human Retinal Pigment Epithelial Cells

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PURPOSE. To determine whether long-term cultured nondividing human retinal pigment epithelial (hRPE) cells are sensitive to oxidant-induced apoptosis and whether the Fas pathway is involved in the process.

METHODS. Confluent hRPE cells were maintained for 2 to 3 months in the basal medium (DMEM containing 2% fetal bovine serum) with one medium change per week. DNA synthesis was measured by incorporation of bromodeoxyuridine (BrdU) and the cell cycle was analyzed by flow cytometry. Intracellular glutathione (GSH) and glutathione disulfide (GSSG) were measured by HPLC. Apoptosis was triggered with the oxidant tert-butylhydroperoxide (tBH), recombinant soluble Fas ligand (sFasL), or agonistic anti-Fas antibody (CH-11). Cell viability was assessed by tetrazolium salt (WST-1) assay, and apoptosis was determined by measuring DNA cleavage or phosphatidylserine exposure. FasL and Fas proteins were detected by flow cytometry and Western blot. FasL and Fas transcripts were analyzed by RT-PCR.

RESULTS. After incubation in basal medium for more than 2 months, hRPE cells were largely nondividing and accumulated autofluorescent granules identified by electron microscopy to be lysosomes. Compared with proliferating hRPE cells, the nondividing cells had lower intracellular GSH, GSSG, and GSH/GSSG and a more oxidized redox potential (Eh). Downregulation of Fas but upregulation of FasL was observed in these cells. The nondividing hRPE cells appeared more susceptible to tBH-induced apoptosis. Similar to proliferating hRPE cells, the apoptosis induced by tBH was preceded by induction of FasL, and antioxidants inhibited both FasL increase and apoptosis. Apoptosis was also inhibited with the antagonistic anti-Fas antibody ZB4. However, the nondividing hRPE cells had decreased sensitivity to apoptosis triggered by sFasL or CH-11.

CONCLUSIONS. Long-term hRPE culture created cells that were nondividing and accumulated autofluorescent granules. The increased sensitivity to tBH-induced apoptosis in these cells was associated with intracellular oxidation and upregulation of FasL. These results suggest that an increase in FasL may contribute to the vulnerability of nondividing hRPE cells to oxidant-induced apoptosis. (Invest Ophthalmol Vis Sci. 2002;43:2546–2553)

A ge-related macular degeneration (ARMD) is the leading cause of blindness in elderly Americans.1 It results from progressive loss of photoreceptors in the central retina, probably due to damage to the underlying retinal pigment epithelium (RPE).2 It has been hypothesized that age-related injury to the RPE is related to oxidative stress.3 The RPE is bathed in a high concentration of oxygen and subjected to intense focused light.4 It also phagocytoses and degrades membranes rich in polyunsaturated fatty acids shed daily from the photoreceptor outer segments.5 In addition, aging RPE is rich in pigments such as lipofuscin.6,7 Studies have shown that lipofuscin is a photoinducible generator of the reactive oxygen species implicated in the general aging process.9 Recently, Suter et al.10 have shown that pyridinium bis-retinoid A2E, a lipofuscin component, induces apoptosis in RPE cells. All these data suggest that oxidative injury of RPE cells may contribute to the pathogenesis of ARMD.

Although RPE cells are subjected to a high metabolic load and to physiological and environmental insults, it is generally believed that they do not undergo appreciable cell division.11 Therefore, adult RPE cells are thought to be quiescent and, after differentiation, remain in G0 for the lifetime of an individual. Nonetheless, RPE cells retain the ability to reenter the cell cycle. They can regenerate to some extent when experimentally damaged in vivo and can proliferate in vitro when placed in culture.12,13 Although cultured RPE cells do not have certain functions that are present in vivo, they preserve enough normal activity that they are widely used in the investigation of questions related to differentiation, survival, injury, fluid transport, and other functions.14

Most studies examining the effects of oxidative stress on RPE have been performed with cultured proliferating RPE cells. Our laboratory has shown that the chemical oxidant tert-butylhydroperoxide (tBH) induces cell death in cultured human RPE (hRPE) cells.15 Glutathione (GSH), its amino acid precursors, and dimethylfumarate, an inducer of GSH synthesis, protect RPE cells from tBH-induced injury.15,16 This injury is caused by apoptosis, with a Fas-mediated pathway involved in tBH-induced damage.15,16

Although these studies have been important in demonstrating the ability to induce oxidative damage to the RPE, the observation in proliferating cells may not reflect accurately apoptosis as it occurs in the nondividing cells in vivo. Hence, in the present study, long-term cultured nondividing hRPE cells were used to study the role of oxidant-induced injury in the RPE.

MATERIALS AND METHODS

hRPE Cell Cultures

Fifteen hRPE cell cultures were established from 15 donor eyes, as previously described.15 Cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS).
2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 37°C humidified incubator in an atmosphere of 5% CO₂ in air. Cells were passed every 7 days. hRPE cells between passages 6 and 10 were used throughout the study.

Generation of Nondividing hRPE Cells
hRPE cells from each established culture were divided into two groups. Cells in the first group were frozen and kept at −86°C. Cells in the second group were seeded in the flasks and grown to confluence in DMEM containing 10% FBS. The growth medium was then replaced with a basal medium (DMEM containing 2% FBS) and the confluent hRPE cells were maintained in this basal medium for over 2 months, changing the medium once a week. One week before experiments, the cells in the first group were thawed and cultured in DMEM containing 10% FBS until they reached logarithmic growth phase. These proliferating hRPE cells were then compared with nondividing hRPE cells generated from the same hRPE culture.

Cell Cycle Analysis
Single hRPE cell suspensions were prepared by trypsinization. After they were washed, cells were fixed and permeabilized with ice-cold 70% ethanol for 30 minutes at −20°C, followed by incubation with 50 μg/mL propidium iodide and 100 μg/mL RNase A for 30 minutes at 37°C in the dark. Data acquisition and analysis were performed in a flow cytometer (FACSscan; BD Biosciences, Mountain View, CA), with the accompanying software (CellQuest; BD Biosciences). Appropriate gating was used to select the easily distinguished single cell population of hRPE cells from the small amount of other debris and aggregates present. The same gate was used on all samples, ensuring that the measurements were made on a standardized cell population.

DNA Synthesis
DNA synthesis was measured by a cell proliferation ELISA bromodeoxyuridine (Brdu) colorimetric kit (Roche Molecular Biochemicals, Indianapolis, IN). hRPE cells were labeled with Brdu for 3 hours at 37°C. After three washes, substrate, tetramethyl-benzidine (TMB), was added and incubated for 20 minutes. A blocking solution (1 M H₂SO₄) was added, and the absorbances of the untreated control were used throughout the study. Dansyl derivatives were detected by fluorescence with 305 to 395 nm bandpass filters. The absorbance of the untreated control.

Apoptosis Assay
DNA cleavage, which commonly occurs in apoptosis, was measured by TdT-mediated dUTP nick-end labeling (TUNEL) with a kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Molecular Biochemicals). Briefly, after treatment, floating and adherent (released by trypsin) cells were collected and fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Label DNA strand breaks, cells were incubated with 50 μL TUNEL reaction mixture containing TdT and fluorescein-dUTP in the binding buffer and incubated for 1 hour at 37°C in a humidified atmosphere. Cells were then washed twice with PBS and analyzed by flow cytometry. Another apoptotic feature, phosphatidylserine exposure, was determined with a kit (Annexin V-FITC Kit TACS; Trevigen, Gaithersburg, MD). Briefly, cells were resuspended in 100 μL binding buffer containing 10 mM HEPES-KOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 1 mg/mL annexin-V-FITC, and propidium iodide. After 15 minutes of incubation at room temperature in the dark, 400 μL binding buffer was added, and cells were analyzed by flow cytometry.

FasL and Fas Expression
Expression of FasL and Fas on hRPE cells were detected, as described previously. For flow cytometric detection of Fasl, cells were sequentially stained with 2.5 μg/mL a rabbit anti-FasL antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 2.5 μg/mL FITC-conjugated anti-rabbit IgG (Pierce, Rockford, IL). For detection of Fas, cells were incubated with phycoerythrin (PE)-conjugated anti-Fas antibody (BD Transduction Laboratories, San Diego, CA). Cells stained with isotypically matched control Ig and FITC-conjugated secondary antibodies and cells stained with PE-conjugated isotypically matched control Ig were run in parallel as negative controls for Fasl and Fas staining, respectively. The cells were then analyzed by flow cytometer. Expression of Fasl was further confirmed by Western blot. Cells were lysed in a boiling solution containing 10% glycerol, 250 mM Tris (pH 8.0), 1% SDS, and 2% β-mercaptoethanol. The cell lysates were immediately boiled for 5 minutes and centrifuged at 10,000g for 5 minutes at 4°C. The supernatants (100 μg) were subjected to 12% SDS-polyacrylamide gel followed by transfer to PVDF membranes (Hybond; Amersham Life Science Inc., Arlington Heights, IL) and immuno blot with anti-Fasl antibody (1:1000 dilution, BD Transduction Laboratories). Fasl protein was detected using horseradish peroxidase labeled secondary antibody and enhanced chemiluminescence (NEN Life Science Products, Boston, MA). Equal protein loading was verified by ponceau red treatment of membranes.

For RT-PCR analysis of Fasl and Fas transcripts, total RNA was prepared with a kit (RNaseasy; Qiagen, Inc., Valencia, CA) and reverse transcribed for 1 hour. Subsequently, 5 μL of the resultant cDNA was subjected to PCR with the use of primers for Fas (forward: 5'-CGG-AGGATTGCTCAACAC-3', reverse: 5'-TTGGTATCTGCTTCTGCG-3').

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Cell Viability
A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (+3-[4,5-iodophenyl]-2H-4-7-tetrazolo]-1-benzene disulfonate) to formazan by mitochondrial dehydrogenases of viable cells was performed (Roche Molecular Biochemicals). hRPE cells were seeded in 96-well plates at a density of 1 × 10⁵ cells/well in 100 μL medium. After 2 days, cells were treated with tBH, recombiant soluble (s)FasL, or agonistic anti-Fas antibody CH-11. The WST-1 solution (10 μL/well) was added, and cells were further incubated for 2 hours at 37°C. The plate was read on an ELISA reader at 450 nm, with a reference wavelength at 650 nm. Data are shown as the percentage of the absorbance of the untreated control.

Measurement of Autofluorescence by Flow Cytometry
hRPE cells were harvested by trypsinization and washed once with PBS. The autofluorescence of 10,000 cells from each sample was then measured at 530 nm (with 30 nm bandwidth) on a flow cytometer, with light excitation at 488 nm.

Electron Microscopy
hRPE cells were trypsinized and centrifuged. The pellets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours at 4°C, followed by postfixation in 1% osmium tetroxide for 1 hour. The cell pellets were then processed for electron microscopy by the standard method.

HPLC Analysis of GSH and GSSG
GSH and GSSG were measured as the S-carboxymethyl-V-dansyl-derivatives by HPLC as previously described. hRPE cells were extracted with ice-cold 5% perchloric acid containing 0.2 M boric acid and 5 μM y-glutamylglutamate (internal standard), treated with iodoacetic acid and dansylchloride, and separated using a propylamine column (Custom LC, Houston, Texas). Dansyl derivatives were detected by fluorescence with 305 to 395 nm bandpass filter for excitation and a 520 nm cutoff filter for emission. Peaks were quantified by integration relative to standards. Redox potential (Eₜₜ) was calculated from the cellular GSH and GSSG concentrations by the Nerst equation and E₀ = −0.252 V at pH 7.2,20,21
and FasL (forward: 5'-GTTTGTGGGGTGGGGTGGACT-3', reverse: 5'-GGAAAGAATCCCAAAGTGCTTC-3'). As a control of reverse transcription and PCR, the following primers: 5'-CGTGGGCCGCCCTAGGCACCA-3' (forward) and 5'-TTGGCCTAGGGTTCAGGGGGG-3' (reverse) were used to amplify h9252-actin. Thirty (Fas and h9252-actin) and 35 cycles (FasL) were performed at 94°C (1 minute), 57°C (1 minute), and 72°C (1 minute). The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

**Statistic Analysis**

Each experiment was performed at least three times with three different pairs of hRPE cells. The student t-test was used to determine whether the means of two different groups are significant. P ≤ 0.05 was accepted as significant. Results are expressed as mean ± SEM.

**RESULTS**

**Nondividing hRPE Cells in Long-Term Culture**

After confluence, hRPE cells were maintained in DMEM containing 2% FBS for more than 2 months by changing medium once a week. Cell cycle analysis revealed that in the long-term culture, 90% of hRPE cells were at G1/G0 phase and approximately 8% cells were in the S and G2/M phases (Fig. 1). In contrast, in hRPE cells grown under the standard condition, only 68% of cells were at the G1/G0 phase and more than 31% of cells were at the S and G2/M phases, indicating that long-term hRPE cells are mostly nondividing. DNA synthesis was analyzed by measuring BrdU incorporation. Significantly less DNA synthesis occurred in long-term cultured hRPE cells than in hRPE cells grown under the standard condition (0.69 ± 0.09 vs 2.49 ± 0.15, n = 5, P < 0.01). Accumulation of autofluorescence granules in long-term cultured hRPE cells was observed in nondividing hRPE cells and, by electron microscopy, the granules were identified to be lysosomes (data not shown).

**Intracellular Oxidation in Nondividing hRPE Cells**

Studies by other investigators have clearly demonstrated that cells become more sensitive to oxidative stress as they cease to divide and differentiate.25,26 To determine whether increased oxidation also occurs in the nondividing hRPE cells, intracellular GSH and GSSG were measured by HPLC. Significant decreases were observed in intracellular GSH, GSSG, and the GSH/GSSG ratio (Fig. 2). In these cells, the calculated GSH/GSSG redox potential was −210 mV, which was more oxidized than that in hRPE cells grown under standard condition (−227 mV). These data show that the GSH pool was oxidized in the nondividing hRPE cells.

**Increased Sensitivity of Nondividing hRPE Cells to Oxidant-Induced Apoptosis**

A previous study showed that proliferating hRPE cells undergo apoptosis after treatment with oxidant tBH.17 To determine whether nondividing hRPE cells were also sensitive to oxidant-induced apoptosis, nondividing cells were treated with various concentrations of tBH for 4 hours, and cell viability was measured. Cell death was also observed in nondividing hRPE cells after tBH treatment (Fig. 3). Compared with the corresponding proliferating hRPE cells, the susceptibility of nondividing hRPE cells to tBH was markedly increased.

**FIGURE 1.** Cell cycle distribution in long-term cultured proliferating (top) and nondividing (bottom) hRPE cells. Cells were trypsinized, fixed with ethanol, and stained with propidium iodide. Cells were then analyzed by flow cytometry. Representative data of three separate experiments are shown. FL2-A, fluorescence intensity of propidium iodide.

**FIGURE 2.** Comparison of redox status in cultured proliferating and nondividing hRPE cells. Intracellular GSH and GSSG contents were measured by HPLC and adjusted to the levels of 1 mg of acid-insoluble protein. Eh was calculated based on GSH and GSSG concentrations and intracellular pH by the Nernst equation. Data are the mean ± SEM of results in five separate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus proliferating hRPE cells.
antioxidants GSH and to oxidant-induced apoptosis. In the nondividing hRPE cells, was found to differ from proliferating cells. Expression of FasL and Fas in the nondividing hRPE cells remained in a long-standing nonmitotic state similar to that formed in RPE cells in vivo that undergo active phagocytosis. Moreover, the autophagocytic components in this long-term hRPE culture may be affected by culture conditions and constituents of the medium and thus differ from that formed in RPE cells in vivo that undergo active phagocytosis. In spite of these disadvantages, cultured hRPE cells are found to regain autofluorescent granules after challenge with isolated lipofuscin or after long-term culture. Accumulation of autofluorescent granules in the cytosol was also observed in our cultures. By electron microscopy, these autofluorescence granules were found to have the appearance of lysosomes. Because hRPE cells were grown without feeding with photoreceptor outer segments, the autofluorescent materials accumulated under this condition were derived from autophagolysosomal components. The autofluorescent components in this long-term hRPE culture may be affected by culture conditions and constituents of the medium and thus differ from that formed in RPE cells in vivo that undergo active phagocytosis. In spite of these concerns, the long-term cultured hRPE cells remained in a long-standing nonmitotic state similar to that occurring in situ during aging in postproliferative tissue and therefore may serve as a better model than proliferating and depigmented RPE cells for studying oxidative injury.

In the nondividing hRPE cells, intracellular oxidation was observed between proliferating and nondividing hRPE cells. Thus, the decrease in Fas-mediated apoptosis may be due to decreased cell surface expression of Fas, but this decrease does not appear to be a direct consequence of decreased mRNA, because no difference in Fas mRNA expression was detectable.

**DISCUSSION**

Human RPE are essentially nonreplicating in vivo. Although regeneration of epithelium can be observed to a limited extent in some experimental situations, cell division in the RPE is usually considered to be abnormal in vivo and, under certain conditions, can contribute to pathologic conditions such as proliferative vitreoretinopathy. Commencing in childhood and continuing throughout life, an autofluorescent pigment, lipofuscin, accumulates in RPE cells. Unlike other long-lived nonmitotic cells in the body, in which lipofuscin is formed through the autophagic breakdown of intracellular organelles, the major source of the lipofuscin in RPE cells is the shed photoreceptor outer segment membranes that are internalized by the RPE daily. A considerable body of evidence has suggested that the accumulation of lipofuscin is related to decreased RPE function and possibly to ARMD.

Developments in tissue culture techniques have led to a success in the establishment of human RPE cell cultures from donor eyes. However, unlike the RPE in vivo, human RPE cells grown in tissue culture continue to proliferate and progressively depigment. The depigmentation seems to occur as a result of redistribution of the initial limited complement of pigmented inclusions among a rapidly increasing number of daughter cells. In spite of these disadvantages, cultured hRPE cells retain enough normal in vivo activities of RPE cell and have made an important contribution in the study of various human retinal diseases. Using cultured hRPE, we have shown previously that the chemical oxidant tBH induces apoptosis in cultured hRPE cells and that this process is mediated by an upregulation of Fas pathway.

In the present study, we used nondividing hRPE cells to study the oxidative mechanism of RPE injury. We found that hRPE cells cultured at confluence for an extended period had decreased DNA synthesis and arrested at G0/G1, indicating that they were mainly nondividing cells. In vitro, cultured RPE cells are found to regain autofluorescent granules after challenge with isolated lipofuscin or after long-term culture. Accumulation of autofluorescent granules in the cytosol was also observed in our cultures. By electron microscopy, these autofluorescence granules were found to have the appearance of lysosomes. Because hRPE cells were grown without feeding with photoreceptor outer segments, the autofluorescent materials accumulated under this condition were derived from autophagolysosomal components. The accumulation of lipofuscin in vivo that have both autophagocytic and heterophagocytic components in this long-term hRPE culture may be affected by culture conditions and constituents of the medium and thus differ from that formed in RPE cells in vivo that undergo active phagocytosis. In spite of these concerns, the long-term cultured hRPE cells remained in a long-standing nonmitotic state similar to that occurring in situ during aging in postproliferative tissue and therefore may serve as a better model than proliferating and depigmented RPE cells for studying oxidative injury.

In the nondividing hRPE cells, intracellular oxidation was indicated by a depletion of the GSH/GSSG pool, a drop in the GSH/GSSG ratio, and oxidation of the GSH/GSSG pool. The oxidation or loss of GSH may be associated with changes of the rate limiting enzyme of GSH synthesis, glutamate-cysteine ligase, the detoxification enzymes, GSH S-transferase and...
NAD(P)H:quinone reductase, and GSH transport across the cell membrane. Indeed, in colon carcinoma cells, intracellular redox potential, as determined by the concentration of GSH and GSSG, becomes more oxidized as cells become differentiated, and this oxidation is associated with activities of GSH S-transferase and NAD(P)H:quinone reductase. In human skeletal muscle cells, differentiation can also decrease the cells' ability to use extracellular GSH and can sensitize the cell to release GSH. Jonas et al. have shown that in malnourished rats, growth of intestinal mucosal cells is inhibited, and it is

**FIGURE 4.** Involvement of the Fas pathway in tBH-induced apoptosis in nondividing hRPE cells. (A) Upregulation of FasL expression by tBH and inhibition by antioxidants. hRPE cells were untreated or treated with the antioxidants GSH (5 mM) and NAC (1 mM) for 1 hour and then incubated with 500 μM tBH. Expression of cell surface FasL was determined after 1 hour by flow cytometry. A single-parameter histogram was plotted as fluorescein intensity, a measure of FasL expression, against cell number. (B) Apoptosis was assessed after 4 hours by examining DNA cleavage with TUNEL. Dot plots show two-parameter analysis of fluorescein intensity (dUTP incorporation) and FSC (indicating cell size). Data show an increase in the cells undergoing apoptosis after tBH treatment (cell shrinkage, indicated by a decrease in cell size, and DNA cleavage, indicated by an increase in fluorescence intensity of dUTP-FITC) and the inhibition by GSH and NAC. (C) Antagonistic anti-Fas antibody ZB4 inhibited tBH-induced apoptosis. hRPE cells were pretreated with 125 ng/mL ZB4 for 1 hour and then treated with 500 μM tBH for 4 hours. Cells were then stained with annexin V-FITC together with propidium iodide and apoptosis was assessed by examining phosphatidylserine (PS) externalization by flow cytometry. Data show two-parameter analysis of fluorescein intensity of annexin V (green fluorescence, PS exposure) and propidium iodide (cell viability). The normal (living) cells (bottom left quadrants) had low annexin V and propidium iodide staining. The early apoptotic cells (top right quadrants) had high annexin V and propidium iodide staining. The late apoptotic cells (bottom right quadrants) had high annexin V and propidium iodide staining. Percentages of cells in each quadrant are indicated within the quadrant. Representative results of three separate experiments are shown.
associated with a decrease of GSH and an increase of GSGG. Refeeding the rats with keratinocyte growth factor causes an increase in GSH level and restores intestinal epithelial growth. This result demonstrates that loss of GSH coinciding with cell differentiation also occurs in vivo. In the present study, non-dividing hRPE cells appeared more sensitive to apoptosis induced by tBH, perhaps due to a depletion of GSH in long-term nondividing hRPE cells. Studies of several cell types have shown that GSH depletion increases the risk of oxidative injury. Refeeding the rats with keratinocyte growth factor causes an increase in GSH level and restores intestinal epithelial growth. This result demonstrates that loss of GSH coinciding with cell differentiation also occurs in vivo. In the present study, non-dividing hRPE cells appeared more sensitive to apoptosis induced by tBH, perhaps due to a depletion of GSH in long-term nondividing hRPE cells. Studies of several cell types have shown that GSH depletion increases the risk of oxidative injury.47–40 Further, exogenous GSH and its amino acid precursors appear to protect these cells from oxidative injury from a variety of toxins, including tBH, paraquat, and menadione.

Because our previous study using proliferating hRPE cells had demonstrated that activation of Fas pathway is involved in tBH-induced apoptosis,18 we studied whether the regulation of the Fas pathway contributes to the increased sensitivity of nondividing hRPE cells to oxidant injury. As observed in proliferating hRPE cells, apoptosis induced by tBH in nondividing hRPE cells was also mediated through the Fas pathway. It was preceded by upregulation of Fasl and inhibition of tBH-induced expression of Fasl by antioxidants eliminated apoptosis caused by tBH. In addition, blocking Fasl and Fas interaction with antagonistic anti-Fas antibody also inhibited tBH-induced apoptosis. Compared with proliferating hRPE cells, nondividing cells expressed a higher level of Fasl and showed a more dramatic increase in Fasl after tBH treatment. It is well known that expression of Fasl is under redox regulation. Studies have shown that reactive oxygen intermediates are involved in the induction of Fasl expression by cytostatic drugs and T-cell activation.41,42 Treatment of cells with pro-oxidants also causes an increase in expression of Fasl.41,42 GSH is the primary source of reducing equivalents in most cells, contributing significantly to the cellular redox potential.43 Depletion of intracellular GSH has been associated with the production of reactive oxygen intermediates and activation of the transcription factors nuclear factor κB (NFκB) and AP-1.44,45 Previous studies have shown that NFκB and AP-1 are involved in the induction of the Fasl gene.45,46 Therefore, it is possible that in nondividing hRPE cells, oxidation of the GSH pool may result in expression of Fasl. However, because there was no increase in Fasl transcript in nondividing hRPE cells, intracellular oxi-

**Figure 5.** Decreased cytotoxicity in nondividing hRPE cells after sFasl and CH-11 treatment. Cells were treated with 500 ng/mL sFasl and 500 ng/mL CH-11 for 48 hours. Cytotoxicity was measured through the cleavage of WST-1. Data points show the mean ± SEM of seven (sFasl) and four (CH-11) separate experiments. ***P < 0.001 versus proliferating hRPE cells.

**Figure 6.** Comparison of FasL and Fas expression in proliferating and nondividing hRPE cells. (A) Flow cytometry measurement of cell surface expression of Fasl and Fas. SMFI = specific mean fluorescence intensity of cells stained with antibody – mean fluorescence intensity of cells stained with isotype-matched Ig. Data are the mean ± SEM of results in 18 (Fasl) or 17 (Fas) separate experiments. *P < 0.05 or **P < 0.001 versus proliferating hRPE cells. (B) Western blot measurement of Fasl expression. (C) RT-PCR analysis of Fasl and Fas transcripts. The PCR products were analyzed by agarose gel electrophoresis. Fasl, Fas, and β-actin migrated at the predicted sizes of 827, 420, and 243 bp, respectively. (B) and (C) show representative results of three separate experiments.
dation may regulate expression of FasL through a posttranscriptional mechanism. In contrast to tBH, the nondoning hRPE cells were less sensitive to apoptosis induced by sFasL and CH-11. This may be due to the downregulation of Fas expression in these cells. There are a variety of effectors and repressors that lie upstream and downstream in the Fas pathway, and these could also affect sensitivity to apoptosis.57

In conclusion, in this study, long-term confluent culture created nondoning hRPE cells. These nondoning hRPE cells were more sensitive to oxidant-induced apoptosis than their proliferating counterparts. Intracellular oxidation and increased expression of FasL may contribute to this increased sensitivity. Therefore, nondoning hRPE cells may be superior to proliferating and depigmented cells for the study of oxidative injury of RPE cells.

References


